REMARKS

The invention is directed at a semen extender composition and a method for manufacturing a semen extender composition. The semen extender composition includes about 0.1 wt.% to about 6 wt.% phospholipid obtained from a non-animal source, an effective amount of surfactant to reduce ice crystal formation during freezing of the composition, a carbohydrate, and a biological buffer to provide a semen extender use solution having a pH of between about 6.9 and about 7.5, and wherein the use solution exhibits an osmolality of about 250 mOsM to about 350 mOsM. The method for manufacturing a semen extender composition includes a step of mixing the extender composition components.

The semen extender composition includes a surfactant to help reduce ice crystal growth during the freezing process and to help strengthen the cell membrane during the freezing and thawing process. Various exemplary surfactants that can be used according to the invention are disclosed by the specification at page 6, lines 4-19. In addition, the surfactant can be used as a single surfactant or as a mixture of surfactant, and can be provided in an amount so that a semen extender composition use solution will include about 0.001 wt.% to about 1 wt.% surfactant.

Prior Art-Based Rejections

The outstanding Office Action includes three prior art-based rejections. These rejections include: (1) a rejection of claims 1-3, 6-8, 11-14, and 21-23 under 35 U.S.C. §102(b) over European Publication No. 0 685 556 (*Ghazarian et al.*); (2) a rejection of claims 1-8, 10-14, and 21-23 under 35 U.S.C. §102(e) over U.S. Patent No. 6,368,786 (*Saint-Ramon et al.*); and (3) a rejection of claims 1-14 and 21-23 under 35 U.S.C. §103(a) over *Ghazarian et al.* or *Saint-Ramon et al.* and U.S. Patent No. 3,444,039 (*Rajamannan*), U.S. Patent No. 6,130,034 (*Aitken*), U.S. Patent No. 6,140,121 (*Ellington et al.*), and C. Hellemann and E. Giroux, Deep Freezing of Rabbit Sperm, Effect of a Surfactant on Fertilizing Capacity, Zuchthyg., 23, 33-37 (1988) (*Hellemann et al.*). These rejections are traversed.

Rejection Over *Ghazarian et al.*

Enclosed with amendment is an English language translation of Ghazarian et al. The discussion of *Ghazarian et al.* that follows will be in reference to the English language translation.

Ghazarian et al. are directed at a vehicle for nonautonomous microorganisms of the animal kingdom to be kept alive outside their natural environment with a view to human interventions. The vehicle includes an aqueous medium comprising nutrition agents, buffers and mineral salts, a protective product formed as support for embryonic growth by a living organism, wherein the protective product is a lecithin extracted from soy seeds and introduced into the aqueous medium upon formation of the vehicle. See the English language translation of *Ghazarian et al.* at page 3, lines 20-27.

Ghazarian et al., however, fail to disclose a semen extender composition containing an effective amount of surfactant to reduce ice crystal formation during freezing of the composition according to the present invention. The outstanding Office Action argues that *Ghazarian et al.* disclose "a mixture of Tris and sodium citrate which act as both surfactant and buffer." This statement is not correct. Enclosed for the Examiner's convenience is a copy of a data sheet from "Life Technologies Catalogue and Reference Guide 2001." This reference identifies "Tris" as "tris(hydroxymethyl) aminomethane." The structure of "Tris" is additionally provided. It is submitted that "Tris" is not a surfactant and does not act as a surfactant. "Tris" is identified by the above-identified patent application as an exemplary pH adjuster, and not as an exemplary surfactant. The Examiner's attention is directed to the above-identified patent application at page 7, line 30 through page 8, line 2. Furthermore, no reason has been provided explaining why one would expect "Tris" to be useful to reduce ice crystal formation during freezing of a semen extender composition according to the present invention.

Because *Ghazarian et al.* fail to disclose a semen extender composition that includes a surfactant in an amount sufficient to reduce ice crystal formation during freezing of the composition, *Ghazarian et al.* do not anticipate the present invention, and withdrawal of the rejection over *Ghazarian et al.* is requested. Furthermore, no reason has been provided why one having ordinary skill in the art would have received a suggestion to modify *Ghazarian et al.* to

include a surfactant in an amount to reduce ice crystal formation during freezing according to the present invention.

Rejection over *Saint-Ramon et al.*

Saint-Ramon et al. disclose a diluent for cryogenic storage of bovine spermatozoa that includes a phospholipid, a liposoluble vitamin accompanied by an emulsifier, an antioxidant, and a polyol. See *Saint-Ramon et al.* at column 1, lines 49-52. According to *Saint-Ramon et al.* at column 1, lines 54-56, the "vitamin is accompanied by an emulsifier, for example sorbitan monooleate, which is sold under the trade name Tween 80." Although *Saint-Ramon et al.* mention a vitamin accompanied by an emulsifier, *Saint-Ramon et al.* fail to teach providing an "effective amount of surfactant to reduce ice crystal formation during freezing of the composition" according to the present invention. According to *Saint-Ramon et al.*, it is the polyol that is "capable of inhibiting the formation of ice crystals." See *Saint-Ramon et al.* at column 2, lines 9-11.

It is recognized that *Saint-Ramon et al.* disclose buffers at, for example, column 2, lines 6-8. *Saint-Ramon et al.*, however, fail to teach providing the buffer so that the resulting semen extender use solution has a pH of about 6.9 to about 7.5 and an osmolality of about 250 mOsM to about 350 mOsM according to the present invention. The outstanding Office Action fails to explain why one having ordinary skill in the art would expect *Saint-Ramon et al.* to provide the pH and osmolality properties according to the present invention.

In view of the above comments, it is submitted that the outstanding Office Action has failed to demonstrate that *Saint-Ramon et al.* disclose the present invention. Accordingly, withdrawal of the rejection under 35 U.S.C. §102(e) is requested.

It is believed that *Saint-Ramon et al.* are not available as prior art against the above-identified patent application. The Applicants expect to file a Declaration under 37 C.F.R. §1.131 demonstrating possession of the invention prior to May 14, 1999, the priority date for *Saint-Ramon et al.*

Rejection over *Ghazarian et al.*, *Saint-Ramon et al.*, *Rajamannan*, *Aitken*, *Ellington et al.*, and *Hellemann et al.*

The outstanding Office Action recognizes that *Ghazarian et al.* and *Saint-Ramon et al.* fail to disclose a semen extender compositions that include various surfactant and antioxidants according to the present invention. *Rajamannan*, *Aitken*, *Ellington et al.*, and *Hellemann et al.* fail to suggest modifying *Ghazarian et al.* and *Saint-Ramon et al.* to achieve the present invention.

Rajamannan discloses a freezable diluent preservative material for live cells that include a water soluble extract of dried egg yolk solids. See the abstract of *Rajamannan*. In contrast, the present invention provides that the phospholipids are obtained from a non-animal source. It is submitted that phospholipids obtained from dried egg yolk solids are phospholipids from an animal source. In general, egg yolk contains many different materials, and no reason has been provided to explain why one having ordinary skill in the art would expect that components useful in a system containing egg yolk would be beneficial in a system containing phospholipids from a non-animal source. Clearly, a composition containing egg yolk is quite different from a composition containing a more purified phospholipid composition obtained from a non-animal source. Accordingly, it is submitted that *Rajamannan* teaches away from the present invention and one having ordinary skill in the art would not have looked to *Rajamannan* to achieve the present invention.

Aitken discloses a cryoprotectant medium containing 20% fresh egg yolk. See *Aitken* at column 1, lines 32-45. Similar to *Rajamannan*, *Aitken* teaches away from using phospholipids obtained from a non-animal source. Accordingly, one having ordinary skill in the art would not have looked to *Aitken* to achieve the present invention.

Ellington et al. disclose the use of a polysaccharide containing arabinose, galactose and/or hexuronic acid in nonspermicidal lubricants and a freezing medium. See the abstract of *Ellington et al.* A cryoprotective medium is disclosed by *Ellington et al.* beginning at column 16, line 55. The medium includes PCAGH (polysaccharides containing arabinose, galactose and/or hexuranic acid, see *Ellington et al.* at column 7, lines 7-10), Tris buffer or sodium citrate buffer for sperm cell, PBS for oocytes or embryos, and a balanced culture medium such as M199

for ESC (embryonic stem cell, Ellington et al. at column 7, lines 63-65). It is understood that M199 and PBS are exemplary salt solutions. See *Ellington et al.* at column 16, lines 13-22.

It is not understood why the outstanding Office Action relies upon *Ellington et al.* *Ellington et al.* clearly fail to disclose or suggest a semen extender composition or a method for manufacturing a semen extender composition according to the present invention, and would not have suggested modifying *Ghazarian et al.* or *Saint-Ramon et al.* to achieve the presently claimed invention.

Hellemann et al. disclose a composition containing egg yolk. The Examiner's attention is directed at page 2 of the English language translation of the article enclosed with this amendment. Clearly, *Hellemann et al.* fail to disclose or suggest a semen extender composition or a method for manufacturing a semen extender composition according to the present invention, and clearly would not have suggested modifying *Ghazarian et al.* or *Saint-Ramon et al.* to achieve the present invention.

In view of the above comments, withdrawal of the prior art-based rejections is requested.

It is believed that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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May 27, 2004

Re: RMTC Job No. 1604-98342

To Whom It May Concern:

This is to certify that a professional translator on our staff who is skilled in the **German** language translated the document(s) noted below from **German** into **English**.

- German Article (Deep Freezing of Rabbit Sperm)

We certify that the attached **English** translation conforms essentially to the original **German** language.

K Vitray

Kim Vitray
Operations Manager

Subscribed and sworn to before me this 27TH day of MAY 2004.

TINA WUELFING
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C. Hellemann[1] and E. Gigoux[1]: Deep Freezing of Rabbit Sperm. Effect of a Surfactant on Fertilizing Capacity[2]

Zuchthyg., 23, 33-37 (1988)

[1] Center for Artificial Insemination, Southern University of Chile, Valdivia, Chile

[2] Received date: May 22, 1987

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DEEP FREEZING OF RABBIT SPERM
EFFECT OF A SURFACTANT ON FERTILIZING CAPACITY

In order to clear, if the protective effect of a Na-lauryl-sulphat containing product (OEP) on acrosomes would increase the fertilyzing capacity of frozen rabbit semen, 19 ejaculates were frozen after dilution with TRIS-extenders containing 4,5% (fraction I), 9,0% (fraction III) dimethyl-sulphoxyde (DMSO) without and with 0,2 % OEP (fractions II and IV). Semen was frozen in 0,25 straws. OEP did'nt have any effect on motility (MOT) of thawed semen, but had a significant effect on acrosome integrity (NAR), compairing both 9,0% DMSO fractions. 50 does were inseminated with each frozen semen fraction and another 50 with fresh semen as controls (fraction V). Conception rates (I=42%, II=34.7%, III=18%, IV=28%, V=52%) and litter size (I=6.0, II=4.8, III=3.3, IV=2.5, V=6.2) indicate that the protective effect of OEP on acrosomes does not or only conditionally increases the conception-rates. A negative effect of OEP on fertilizing capacity of sperm could not be stated.

The importance of the evolution of the acrosomes as a spermatological parameter, which was first incorporated by Weitze et al. (1975), was corroborated in continuing experiments to the extent that there is a significant relationship between the fraction of normal acrosomes in deep frozen (DF) sperm and the number of fetuses born per insemination (Helleman, 1976), or that a minimum number of "intact" spermatozoa in the insemination dose is necessary for undisturbed fertilization (Weitze, 1977; Weitze 1981). Weitze, et al. (1975) called attention to the acrosome-damaging effect of the cryoprotector dimethylsulfoxide (DMSO) in the deep freezing of sperm, according to comparative tests with end concentrations of 2.5% and 4.5% DMSO in the semen extender. This effect was confirmed in comparative tests of extenders containing 2%, 4.5%, 7% and 9% DMSO, where a clearly elevated number of damaged acrosomes was found, in particular with 9% DMSO in comparison to 2% DMSO (Hellemann, 1976). However, in the same tests, the acrosome damage could be significantly reduced through the addition of 0.2% OEP*, a surface-active detergent. However, a clear effect on the insemination results could not be detected in a preliminary insemination experiment. The goal of this work was to investigate the effect of added OEP on the spermatological parameter motility (MOT) and the acrosome integrity in expanded deep freezing tests and to verify in an insemination test whether the fertilizing capacity of the DF sperm can be increased through the addition of OEP.

Material and methods

The semen donors were 9 Angora bucks, which were used at an insemination station to provide the insemination service with fresh sperm. The test inseminations were carried out in

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* OPE = "Orvus ES Paste." Na lauryl sulfate and other organic components. New name: "EQUEX STM" Nova Chemical Sales, Inc., P. O. Box 144, Scituate, Mass. 02066.

commercial angora breeding operations. There were 19 ejaculates available for processing, which were divided into 4 fractions and diluted at room temperature as single phase to about 3 to 5 million sperm cells per dose with the extenders indicated in Table 1, filled into 0.25-mL straws and packed in aluminum round cassettes, and then, after 2 hours adaptation time, frozen at +5°C in nitrogen vapor 2 cm above the nitrogen level. One thawed sample per ejaculate was tested for motility (MOT) and, after being fixed with a physiological salt solution containing 0.3% NaF, the fraction of undamaged acrosomes was counted by contrast microscope evaluation of the normal apical edge (NAE). Of the deep frozen ejaculates, 4 were used for the test inseminations. 250 animals were inseminated, alternating one doe per fraction in each case, followed by a 5th control insemination with fresh sperm. To initiate ovulation 0.2 mL of the synthetic GnRH preparation Receptal® was injected i.m. immediately after each insemination.

Table 1: Composition of extenders comparatively used for freezing rabbit semen

(1) FRAKTION	1	2	3	4
Stammlösung* (2)	74,0 ml	74,0 ml	74,0 ml	74,0 ml
DMSO	4,5 ml	4,5 ml	9,0 ml	9,0 ml
OEP	–	0,2 ml	–	0,2 ml
Aqua dest.	4,5 ml	4,5 ml	–	–
(3) Eigelb	17,0 ml	17,0 ml	17,0 ml	17,0 ml

* Stammlösung: (2)	
(4) TRIS (hydroxymethyl)-aminomethan	2,523 g
DGlucose	1,042 g
Citronensäure anh. (5)	1,276 g
Aqua dest. ad.	75,5 ml
(6) Glycerin	0,5 ml

[Editor's note: In figures and tables, commas in numbers represent decimal points.]

Key:
1 Fraction
2 Parent solution
3 Egg yolk
4 Tris(hydroxymethyl)aminomethane
5 Anhydrous citric acid
6 Glycerol

The measured values of the spermatological parameters MOT and NAE were graphically plotted as the accumulated polygon curve of the frequency distribution (Lorenz, 1984) and the test differences were statistically verified by the Kruskal-Wallis test. Conception rates, litter size and number of fetuses born were subjected to chi^2 tests. In all cases, an error probability of 5% applied.

Results

a) Effect of OEP on the parameters MOT and NAE.

The distribution of the measurement MOT values of the 19 frozen ejaculates can be seen in Figure 1. The MOT values represented there did not show significant differences between the extenders that were used. It is also evident that, in 70-80% of the samples, tested the estimated MOT values lay chiefly between 10 and 30%.

The distribution of the measured NAE values of the same ejaculates follows in Figure 2. From this, one can see that, in the case of the 9.0% DMSO fraction, about 40% of the samples showed under 5% NAE and none were over 20%. The difference to the 3 comparison fractions is significant.

b) Effect of OEP on the fertilization parameters

The results of the test inseminations are given in Table 2. This table shows that the 4.5% DMSO fraction came closest to the fresh sperm fraction both with regard to the conception rate and the litter size. In the case of the 9.0% DMSO fractions, the litter sizes and animals born per insemination were significantly lower than those for the fresh sperm fraction, and in some cases even the 4.5% DMSO fraction.

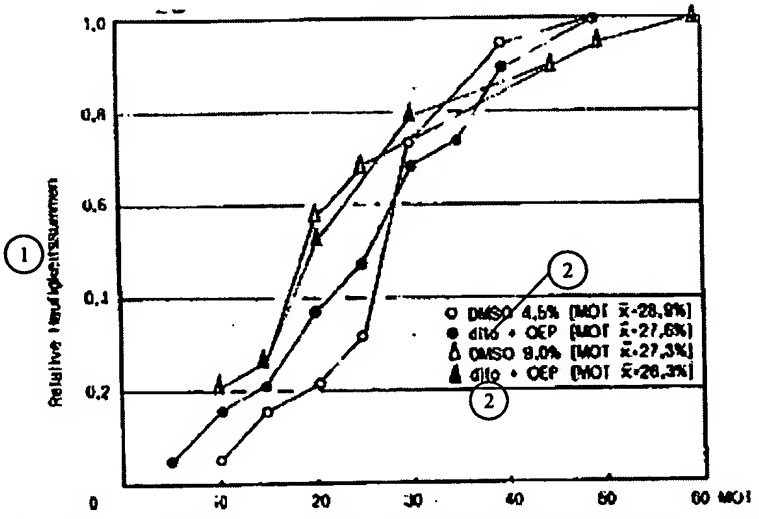

Fig. 1: Accumulated distribution of motility-rates (MOT) in rabbit semen frozen with extenders containing 4.5% and 9.0% dimethyl-sulphoxide (DMSO), comparatively added with 0.2% OEP (n=19).

Key: 1 Relative frequency totals
2 Ditto

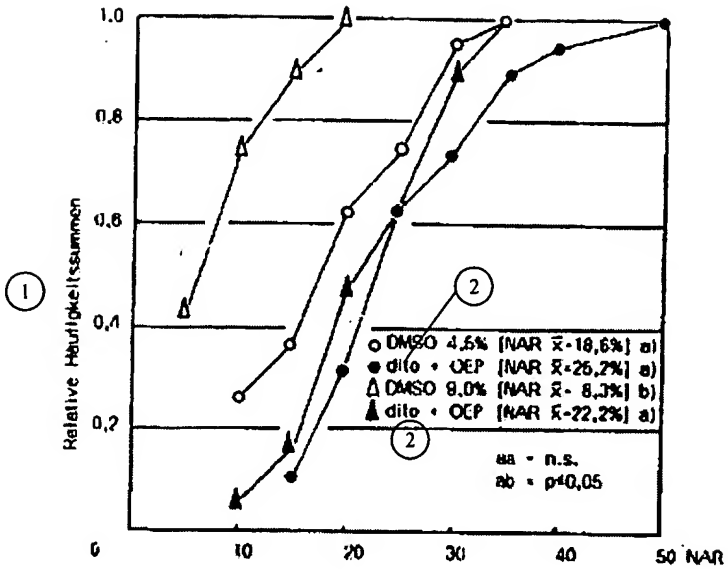

Fig. 2: Accumulated distribution of spermatozoa with normal acrosomal ridge (NAR) in rabbit semen frozen with extenders containing 4.5% and 9.0% dimethyl-sulphoxide (DMSO), comparatively added with 0.2% OEP (n=19).

Key: 1 Relative frequency totals
2 Ditto

Table 2: Conception-rates, litter size and young born by insemination with fresh and frozen rabbit semen containing 4,5% and 9,0% dimithyl-sulphoxide (DMSO), comparatively added with 0,2% OEP

(1) FRAKTION	I 4,5% DMSO	II 4,5% DMSO +0,2% OEP	III 9,0% DMSO	IV 9,0% DMSO +0,2% OEP	(3) FRISCH-SPERMA V
	(2) TG-SPERMA				
(4) Besamungen (n)	50	49	50	50	50
(4) Konzeptionsrate (%)	42,0 a	34,7 a	18,0 b	28,0 a	52,0 a
(4) Wurfgröße ($\bar{X}$)	6,0 a	4,8	3,3	2,5 b	6,2 a
(4) Früchte/Besamung	2,5 a	1,7	0,3 b	0,7 c	3,2 a

(5) aa = nicht signifikant
ab = $p < 0,05$
ac = $p < 0,05$

Key: 1 Fraction
2 DF sperm
3 Fresh sperm
4 Inseminations
Conception rate
Litter size
Fetuses/insemination
5 aa = not significant

Discussion

The expected motility differences were not detected in the comparison of the DMSO concentrations of 4.5% and 9.0%, in contrast to earlier studies, where a tendency toward an elevation of motility with increasing DMSO concentration was evident in a comparison of extender containing 2.0%, 4.5%, 7.0% and 9.0% DMSO (Helleman, 1976) (Figure 1). One can also see from Figure 1 that the addition of OEP did not have any effect on motility. In contrast, one can clearly see a distinct protective effect on the acrosome integrity by OEP, which produced a significant difference between the 9.0% DMSO fractions (Figure 2), where the NAE values for the 9.0% DMSO fraction + OEP reached approximately the same level as the other fractions.

Thus, according to the microscope tests, the spermatological parameters MOT and NAE for fractions I, II and IV were nearly comparable, and, as expected, would probably also produce comparable fertilization results. However, it can be seen from the evaluation of the fertilization parameters in Table 2 that the increase of the NAE values produced by OEP were not or were only conditionally accompanied by an elevation of the fertilization results. Only in the comparison of the two 9.0% DMSO fractions could the higher conception rate be ascribed to the effect of OEP. The addition of OEP to the 4.5% DMSO fraction did produce lower values in the conception rate and litter size, but the deviations could not be statistically confirmed.

All in all, the achieved conception rates appear to be rather moderate, even for the control inseminations with fresh sperm. However, these achieved similar percentages as the approximately 2000 routine inseminations conducted in the same time period, late autumn 1986, using fresh sperm. Coats that were too long for the reproduction performance at the time of the insemination, as already described by Brockhausen et al. (1985), and day/night light ratios not matched to the season in the cages (Lange, 1984) of most breeding operations were found and probably contributed as factors to the less than completely satisfactory results. Finally, it can be concluded from the results that the acrosome-protecting effect of OEP is not or is only conditionally accompanied by an increase of the fertilization capacity of the sperm. A reduction of the fertilization capacity of the sperm produced by the addition of OEP could not be statistically confirmed.

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RALPH
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May 27, 2004

Re: RMTC Job No. 1604-98343

To Whom It May Concern:

This is to certify that a professional translator on our staff who is skilled in the **French** language translated the document(s) noted below from **French** into **English**.

- European Patent 0 685 556 A1

We certify that the attached **English** translation conforms essentially to the original **French** language.

Kim Vitray
Operations Manager

Subscribed and sworn to before me this 27TH day of MAY 2004.

TINA WUELFING
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My Commission Expires
December 8, 2007

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AQUEOUS VEHICLE FOR NONAUTONOMOUS MICROORGANISMS OF THE ANIMAL KINGDOM TO BE KEPT ALIVE OUTSIDE THEIR NATURAL ENVIRONMENT, ESPECIALLY BOVINE SPERMATOZOA

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The vehicle of the invention is designed for nonautonomous microorganisms of the animal kingdom such as gametes or embryos that need to be kept alive outside their natural environment with a view to human interventions, and is capable of being stored ready to use over prolonged periods. It concerns an aqueous medium that comprises, especially beside nutrition agents (sugars and amino acids), buffers and mineral salts usually used, a protective product formed as support for embryo growth by a living organism, which, in the state of the technique is of animal origin and is added immediately before use of the vehicle and which, according to the invention, is a lecithin extracted from soy seeds and introduced into the aqueous medium upon formulation of the vehicle. When the storage of the microorganisms is cryogenic, glycerol is added to the vehicle. In a specific application, the microorganisms are bovine spermatozoa.

The invention is related to a vehicle for nonautonomous microorganisms of the animal kingdom, such as gametes and embryos, for keeping them alive outside their natural environment with a view to human intervention, capable of being stored ready to use over prolonged periods, this vehicle being an aqueous medium comprising, especially beside nutrition agents, buffers and mineral salts, a protective product formed as support for embryonic growth by a living organism and which, in the state of the technique is of animal origin and added immediately before use of the vehicle.

In the sense of the present description, nonautonomous microorganism is understood to be unicellular or paucicellular, eukaryotes or prokaryotes, living but without the ability for immediate reproduction; that is, for reproduction of descendent organisms at the same stage of evolution as their direct ancestors. Thus, gametes or embryos are not autonomous because they cannot directly create gametes or embryos. On the other hand, amebas form autonomous microorganisms.

The storage of living gametes, embryos and cells of the animal kingdom outside their natural environment in the fresh state and especially frozen for veterinary or closely related interventions, especially for artificial fertilization, requires liquid vehicles which in terms of the expert are called "diluents" or "preservatives" for spermatozoa and media for oocytes, embryos and cells. These liquids include in aqueous media, nutrition agents such as sugars and amino acids, buffers and possibly anticoagulants, as well as, for cryogenic storage of microorganisms, an inhibitor of ice crystal formation such as a polyol, especially glycerol. To it is added at the time of use a protective product for cell membranes that is of animal origin such as serums and

albumins; very often this protective product for the diluents is egg yolk, the protective ability of which is particularly accentuated because, it seems, that this egg yolk forms the essential support of growth of the chicken embryo; therefore, of a fragile organism at a stage where cell division is particularly intense, and the defense reactions to external injury even less developed. In practice, either fresh egg yolk or industrial liquid preparations designed especially for the food industry are used.

In compensation for the effectiveness of membrane protection, these protective products are favorable for the multiplication of microorganisms that may or may not be pathogenic, their storage in the effective state is temporary such that they are systematically added to the vehicle only at the time of use.

But this addition at the time of use is an operation that is very difficult to carry out under rigorous aseptic conditions, especially as the sterilization of the protective products is very tricky, because it must neither deteriorate the protective product itself (the products containing albuminoids coagulate with heat, for example) nor allow toxic compounds to exist in the sterilized stored protective product for the microorganisms.

The objective of the invention is therefore to create a vehicle for nonautonomous microorganisms of the animal kingdom stored alive, capable of being stored over prolonged periods ready to use; that is, containing the protective product from the time of formulation, and sterilized.

That is obtained with a vehicle for nonautonomous microorganisms of the animal kingdom, such as gametes or embryos, to be kept alive outside their natural environment with a view to human interventions, capable of being stored ready to use over prolonged periods, this vehicle being an aqueous medium comprising, especially besides nutrition agents, buffers and mineral salts, a protective product formed as support for embryonic growth by a living organism, and which in the state of the technique, is of animal origin and added immediately before use of the vehicle, characterized in that said protective product is a lecithin extracted from soy seeds, and introduced into the aqueous medium upon formulation of the vehicle.

It has been noted that, unpredictably, lecithin extracted from soy seeds, although derived from the plant kingdom and forming part of the support for development of the embryo of a seed, displays a protective power comparable to that of the usual protectors with regard to cell membranes belonging to the animal kingdom, without sharing their fragility in the face of the sterilization processes and poor storage over time, which made it possible to incorporate it in the vehicle during formulation of the latter, and to sterilize the vehicle with all its components. Under these conditions, the vehicle could be stored at refrigerator temperature (typically 4 °C) for prolonged periods, attaining at the least six months, without detectable deterioration.

Preferably, for cryogenic storage of nonautonomous microorganisms, the vehicle will contain a polyol at effective dose that is capable of inhibiting the formation of ice crystals, typically glycerol. The use of such a polyol, standard in cryogenic storage, proved to be compatible with the vehicle of the invention.

Also preferably, the vehicle is formulated with a reduced amount of water, and it is diluted with sterile water for use. Thus, the volume of the vehicle is reduced during storage between its formulation and its use; the provision of sterile water and the dilution of the concentrated vehicle without affecting the sterility of the final vehicle do not present any particular difficulty comparable with the prior practice of addition of the protective product immediately before use.

In a preferred arrangement, a vehicle especially designed for bovine sperm comprises in the concentrated state for 200 mL of water:

Trimethylol methylamine	3.4 g to 4.2 g
Trisodium citrate dihydrate	13.7 g to 16.75 g
Potassium chloride	0.55 g to 0.67 g
Fructose	1.65 g to 2.0 g
Glucose	0.68 g to 0.84 g
Lactose	0.41 g to 0.50 g
Calcium lactate	0.09 g to 0.11 g
Glycine	5.15 g to 6.25 g
Glycerol	64 mL to 78 mL
Soy lecithin	6.75 g to 8.25 g

this vehicle being diluted for use with 750 mL to 900 mL of water.

Secondary characteristics and advantages of the invention will moreover emerge from the description that follows by way of example.

Example 1 – Vehicle for bovine sperm

A vehicle is formulated by dissolving or dispersing in 200 mL of sterile water 3.809 g of trimethylol methylamine buffer (Tris buffer), 15.238 g of trisodium citrate dihydrate anticoagulant, 0.609 g of potassium chloride, of sugars, 1.828 g of fructose, 0.761 g of glucose and 0.457 g of lactose, 0.100 g of sodium lactate, 5.714 g of an amino acid, here glycine, 71 mL of glycerol and 7.5 g of soy lecithin. For use, the quantity of water will be brought to 1025 mL by addition of 825 mL of sterile water.

After formulation, the concentrated vehicle is sterilized in the usual way, put in a tight bottle and stored in a refrigerator at +4 °C.

Example 2 – Use of the vehicle

In the laboratory, bull sperm is frozen, each ejaculate being divided into two parts; one part was introduced into a vehicle in conformance with Example 1 after dilution, and the other part was introduced into a diluent with a formulation owned by the Applicant, including milk and egg yolk. In a standard way, to each part was added the usual antibiotics in equivalent proportions. After which, both parts were put separately into tubes called straws which were sealed and immersed in liquid nitrogen, according to a standard process.

Example 3 – Monitoring of the quality of storage of the spermatozoa in vitro

An equal number of straws belonging to both parts of the ejaculates from several bulls (to reduce the influence of the individual), and chosen at random, were removed from the liquid nitrogen and thawed for microscopic examination, while determining the percentages of motile and progressive spermatozoa. These percentages are revealed to be of the same order for the standard diluents and that according to the invention, with an advantage for the diluent according to the invention; but this advantage is not significant in terms of probability.

Example 4 – Monitoring by in vivo fertilization

Two lots of straws are chosen as with Example 3 that belong respectively to both parts, to fertilize in vitro oocytes sampled from cows and the number of oocytes fertilized is counted as well as the number of embryos that reached the blastocyst stage. There again the percentages of success proved to be approximately equal for both lots, with an advantage for the lot comprising the diluent of the invention. However, on account of the dispersion resulting from the diversity of oocytes, the advantage was not significant in terms of probability.

It will be observed that the preceding examples were designed to verify that the diluent of the invention was at least as effective as the standard diluents, and could stress a significant advantage of the invention, which is to operate routinely under sterile conditions. In fact, on the one hand, the laboratory tests may be carried out with more care and in a better defined environment than work of an industrial nature, and on the other hand, the risk of contaminations created by the standard processes practically do not appear in the early stages surrounding the fertilization.

In another aspect, it has been verified that the concentrations of vehicle components in a range of ±10% around values given in Example 1 do not appreciably degrade the effectiveness of the vehicles with respect to the spermatozoa. It will be observed that glycerol and lecithin contribute less than the other components to the biological compatibility of the vehicle for the nonautonomous microorganisms; glycerol being from this point of view approximately neutral

and acting only to prevent bursting of the membranes when frozen whereas lecithin appears as an external protector for microorganisms.

Moreover, trials with composition of media for storage of oocytes have been made with a view to fertilization "in vitro" and culture of embryos before implantation in the uterus of females, starting from the usual media compositions and adding the soy lecithin formulation to them in substitution for the usual protective products added immediately before use, with results from the point of view of maintaining the life of the nonautonomous microorganisms at least comparable to the results usually obtained, whereas the aseptic conditions of the processes were clearly improved.

Of course, the invention is not limited to the examples described, but embraces all variants of execution within the scope of the claims.

Claims

1. Vehicle for nonautonomous microorganisms of the animal kingdom, such as gametes or embryos, to be kept alive outside their natural environment with a view to human interventions, capable of being stored ready to use over prolonged periods, this vehicle being an aqueous medium comprising, especially beside nutrition agents, buffers and mineral salts, a protective product formed as support for embryonic growth by a living organism, and which in the state of the technique, is of animal origin and added immediately before use of the vehicle, characterized in that said protective product is a lecithin extracted from soy seeds, and introduced into the aqueous medium upon formulation of the vehicle.

2. Vehicle according to Claim 1 provided for cryogenic storage of nonautonomous microorganisms characterized in that at an effective dose it contains a polyol capable of inhibiting the formation of ice crystals.

3. Vehicle according to one of Claims 1 and 2 characterized in that for storage between formulation and use, the concentrated vehicle includes a partial amount of water, a complement of water to be added for use.

4. Vehicle according to Claim 3 specially designed for bovine sperm, characterized in that in the concentrated state it comprises 200 mL of water:

Trimethylol methylamine	3.4 g to 4.2 g
Trisodium citrate dihydrate	13.7 g to 16.75 g
Potassium chloride	0.55 g to 0.67 g
Fructose	1.65 g to 2.0 g
Glucose	0.68 g to 0.84 g
Lactose	0.41 g to 0.50 g
Calcium lactate	0.09 g to 0.11 g

Glycine	5.15 g to 6.25 g
Glycerol	64 mL to 78 mL
Soy lecithin	6.75 g to 8.25 g

this vehicle being diluted for use with 750 mL to 900 mL of water.

5. Vehicle according to Claim 4 characterized in that it contains approximately for 200 mL of water:

Trimethylol methylamine	3.809 g
Trisodium citrate dihydrate	15.238 g
Potassium chloride	0.609 g
Fructose	1.828 g
Glucose	0.761 g
Lactose	0.457 g
Calcium lactate	0.100 g
Glycine	5.714 g
Glycerol	71 mL
Soy lecithin	7.5 g

this vehicle being diluted for use with approximately 825 mL of water.

European Patent Office

Application Number EP 95 40 1170

EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.[6])
X	THE JOURNAL OF PROTOZOOLOGY, vol. 17, No. 2, May 1970, pages 151-152, D. COX "Prolonged survival of Tetrahymena at 0.5°C in Citrated, Lecithinized, defined media." *abstract*	1-5	C12N 5/06
X	TRANSACTIONS OF THE AMERICAN FISHERIES SOCIETY, vol. 112, 1983, pages 86-94, J.H. KERBY "Cryogenic preservation of sperm from Striped Bass." *the entire document*	1-5	
X	GAMETE RESEARCH, vol. 17, 1987, pages 355-373, A.M. SIMPSON ET AL. "Susceptibility of epididymal Boar sperm to cold shock and protective action of Phosphatidylcholine." *the entire document*	1-5	TECHNICAL FIELDS SEARCHED (Int. Cl.[6]) C12N C12Q
A	EP.A.0 521 674 (T. DOMINKO) January 7, 1993		
A	EP.A.0 559 307 (W.R. GRACE & CO. -CONN) September 8, 1993		
A	FR.A.2 350 395 (MC DONNELL DOUGLAS CO.) December 2, 1997		
The present search report has been drawn up for all claims.			

Place of search	Date of completion of the search	Examiner
The Hague	July 18, 1995	Cartagena y Abella, P.

CATEGORY OF CITED DOCUMENTS

X: Particularly relevant if taken alone.
Y: Particularly relevant if combined with another document of the same category.
A: Technological background.
O: Non-written disclosure.
P: Intermediate document.

T: Theory or principle underlying the invention.
E: Earlier patent document, but published on, or after the filing date.
D: Document cited in the application.
L: Document cited for other reasons.

&: Member of the same patent family, corresponding document.

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ULTRA PURE REAGENTS

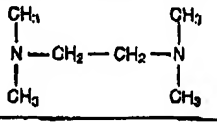
Product	Cat. No.	Size	Quantity	Price
TEMED	15524-010	30 ml	—	$25.00

TEMED (N,N,N′,N′-tetramethylethylenediamine) (Ultra Pure) is the most commonly used accelerator for the polymerization of acrylamide gels.

Analytical Specifications

molecular weight	116.21
appearance	clear liquid
purity	≥99%
refractive index	1.417 ± 0.002

TEMED



Recommended storage condition: 2°C to 8°C, dark.

Attention: This Material is hazardous. All persons using this product should review the Material Safety Data Sheet before handling.

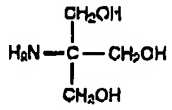
Tris				
	15504-012	500 g	—	$34.35
	15504-020	1 kg	—	64.90
	15504-038	5 kg	—	234.15

Tris [tris(hydroxymethyl)aminomethane] (Ultra Pure) is widely used in buffers because of its buffering range (pH 7.0 to 9.0) and compatibility with many enzymes, including restriction endonucleases and DNA-modifying enzymes.

Analytical Specifications

molecular weight	121.14
appearance	white, free-flowing crystals
purity	≥99.9%
melting point	169°C to 173°C
A_{260} of 1 M Tris	≤0.06
A_{280} of 1 M Tris	≤0.2 (A_{280} < 0.05)
moisture	≤0.3%
calcium	≤1 ppm
lead	<2 ppm
magnesium	<1 ppm

Tris



Performance and quality testing: No detectable contaminating activity is observed in DNA nicking, ribonuclease, and protease assays.

Recommended storage condition: 15°C to 30°C.

Attention: This Material is hazardous. All persons using this product should review the Material Safety Data Sheet before handling.

1 M Tris-HCl Buffers

1 M Tris-HCl, pH 7.2	15566-029	1 L	—	$27.05
1 M Tris-HCl, pH 7.5	15567-027	1 L	—	27.05
1 M Tris-HCl, pH 8.0	15568-025	1 L	—	27.05

1 M Tris-HCl Buffers (Ultra Pure) are premixed and pH-adjusted solutions. Prepared as 1 M concentrates, these buffers can be diluted to the desired concentration and used in molecular biology or general biochemistry applications.

Analytical Specifications

Solution	pH 8.0	pH 7.5	pH 7.2
pH of the 1 M solution at 25°C	8.00 ± 0.10	7.50 ± 0.10	7.20 ± 0.10
pH of the 0.5 M solution at 25°C	8.05 ± 0.10	7.56 ± 0.10	7.27 ± 0.10
pH of the 0.1 M solution at 25°C	8.07 ± 0.10	7.58 ± 0.10	7.29 ± 0.10

Performance and quality testing: No detectable contaminating activity is observed in DNA nicking, ribonuclease, and protease assays.

Recommended storage condition: 2°C to 8°C.

Hazard warning: Irritant.